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RESEARCH PAPER

Long-term consequences of perinatal fatty acid amino hydrolase inhibition

Chia-Shan Wu[1,2,3]*, Daniel Morgan[4]*, Chris P Jew[1,2,3,5], Chris Haskins[4], Mary-Jeanette Andrews[4], Emma Leishman[4], Corinne M Spencer[2,6], Traci Czyzyk[4], Heather Bradshaw[4], Ken Mackie[4] and Hui-Chen Lu[1,2,3,5,7]

[1]*The Cain Foundation Laboratories, Baylor College of Medicine, Houston, TX, USA,* [2]*Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital, Baylor College of Medicine, Houston, TX, USA,* [3]*Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA,* [4]*Department of Psychological & Brain Sciences, Indiana University, Bloomington, IN, USA,* [5]*Program in Developmental Biology, Baylor College of Medicine, Houston, TX, USA,* [6]*Department of Genetics, Baylor College of Medicine, Houston, TX, USA, and* [7]*Department of Neuroscience, Baylor College of Medicine, Houston, TX, USA*

Correspondence
Hui-Chen Lu, Baylor College of Medicine, The Cain Foundation Laboratories/Jan and Dan Duncan Neurological Research Institute, 1250 Moursund St. Suite 1225, TX 77030, Houston.
E-mail: hclu@bcm.edu

*these authors contributed equally to this work.

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BACKGROUND AND PURPOSE
Fatty acid amide hydrolase inhibitors show promise as a treatment for anxiety, depression and pain. Here we investigated whether perinatal exposure to URB597, a fatty acid amide hydrolase inhibitor, alters brain development and affects behaviour in adult mice.

EXPERIMENTAL APPROACH
Mouse dams were treated daily from gestational day 10.5 to 16.5 with 1, 3 or 10 mg kg^{-1} URB597. MS was used to measure a panel of endocannabinoids and related lipid compounds and brain development was assessed at embryonic day 16.5. Separate cohorts of mouse dams were treated with 10 mg kg^{-1} URB597, from gestational day 10.5 to postnatal day 7, and the adult offspring were assessed with a battery of behavioural tests.

KEY RESULTS
Perinatal URB597 exposure elevated anandamide and related *N*-acyl amides. URB597 did not induce signs of toxicity or affect dam weight gain, neurogenesis or axonal development at embryonic day 16.5. It did lead to subtle behavioural deficits in adult offspring, manifested by reduced cocaine-conditioned preference, increased depressive behaviours and impaired working memory. Anxiety levels, motor function and sensory-motor gating were not significantly altered.

CONCLUSIONS AND IMPLICATIONS
Taken together, the present results highlight how exposure to elevated levels of anandamide and related *N*-acyl amides during brain development can lead to subtle alterations in behaviour in adulthood.

LINKED ARTICLES
This article is part of a themed section on Cannabinoids 2013. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-6

Abbreviations
Δ9-THC, Δ9-tetrahydrocannabinol; 2-AG, 2-arachidonoylglycerol; AEA, N-arachidonoyl ethanolamide; CB1 receptor, cannabinoid receptor 1; E, embryonic day; eCBs, endocannabinoids; FAAH, fatty acid amide hydrolase; PPI, prepulse inhibition; URB597, [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate

Introduction

The endocannabinoid system modulates many physiological processes ranging from synaptic function in the CNS to metabolic effects in the periphery (Freund *et al.*, 2003; Osei-Hyiaman *et al.*, 2005; Tam *et al.*, 2006; Harkany *et al.*, 2007; Katona and Freund, 2008; Kano *et al.*, 2009). The endocannabinoid system is comprised of endocannabinoids (eCBs), the metabolic enzymes responsible for the formation and degradation of eCBs, and cannabinoid receptors and their interacting proteins (Piomelli, 2003; Mackie, 2006). Inhibitors that slow the degradation of the eCB *N*-arachidonoyl ethanolamine (AEA), also known as anandamide have the potential to treat anxiety, depression and pain (Lichtman *et al.*, 2004a; Bortolato *et al.*, 2007; Ahn *et al.*, 2009; Guindon *et al.*, 2013). These inhibitors block the hydrolysis of AEA by fatty acid amide hydrolase (FAAH), leading to enhanced eCB signalling, often through cannabinoid receptor 1 (CB_1) (Lichtman *et al.*, 2004b; Piomelli *et al.*, 2006; Ahn *et al.*, 2007; Johnson *et al.*, 2009), although it could also occur through other acyl amide targets such as TRPV1, GPR18, GPR119 and the PPARs (Zygmunt *et al.*, 1999; Lo Verme *et al.*, 2005; Kohno *et al.*, 2006; Overton *et al.*, 2006).

It is now well documented that eCB signalling plays a central role in brain development (Harkany *et al.*, 2007; 2008; Galve-Roperh *et al.*, 2009; Keimpema *et al.*, 2011; Wu *et al.*, 2011). Perinatal and adolescent *Cannabis* exposure may disrupt the precise temporal and spatial control of eCB signalling at critical stages of neural development, leading to detrimental effects later on nervous system functioning (Wu *et al.*, 2011). Indeed, longitudinal studies in humans with prenatal *Cannabis* exposure have shown that as infants they have an exaggerated startle response, exhibit poor habituation to novel stimuli and are hyperactive, and, as adolescents, have inattention and impaired executive function (Richardson *et al.*, 1995; Fried *et al.*, 2003; Jutras-Aswad *et al.*, 2009; Schneider, 2009). AEA participates in multiple aspects of neurodevelopment including neurogenesis, neuronal migration and axonal pathfinding (Keimpema *et al.*, 2011; Wu *et al.*, 2011; Gaffuri *et al.*, 2012). Several studies have found that genetic or pharmacological disruption of endocannabinoid signalling during mouse embryogenesis leads to abnormal patterns of development in the CNS (e.g. neurogenesis, neuronal migration and axonal pathfinding) (Mulder *et al.*, 2008; Jutras-Aswad *et al.*, 2009). While the acute toxicity of FAAH inhibitors has been studied and appears to be mild (Piomelli *et al.*, 2006; Long *et al.*, 2009), the long-term effects of FAAH inhibitors, particularly on the brain as it develops, have not been reported.

The current study aimed to determine if therapeutically active doses of FAAH inhibitors increase embryonic brain levels of AEA and related *N*-acyl amides, and if this was sufficient to impair neurodevelopment and to have a long-lasting effect on CNS function. Firstly, MS was used to determine if the administration of a therapeutic dose of the FAAH inhibitor [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (URB597) increases AEA and related *N*-acyl amide levels in the developing brain. Possible effects of prenatal exposure to URB597 on neurogenesis and axonal development were also assessed. Next, the question of whether administration of URB597 during the perinatal period led to a sustained impairment of behaviour during adulthood, was addressed. Mice were exposed to URB597, at a dose that effectively increased AEA levels, through their mothers from the mid-embryonic period through to the first 7 postnatal days, which approximates the period of maximal neurodevelopment in the human fetus (Marin-Padilla, 1988; Clancy *et al.*, 2001). These mice were then tested as adults with a panel of behaviour assays to assess their anxiety, motor function, sensory-motor gating, drug preference, learning and memory. Our findings indicate that perinatal exposure to an FAAH inhibitor leads to large increases in embryonic AEA and other *N*-acyl amides, and alterations in a restricted set of behaviours in the adult mouse.

Method

Animals and drug treatment

ICR mouse colonies were maintained in a pathogen-free environment with a 14:10 h light : dark cycle with access to food and water *ad libitum*. Pregnant dams (3–5 months of age, non-virgin) used for drug treatment were obtained by timed mating. The day of vaginal plug was designated as embryonic day (E) 0.5 for the embryos and the day of birth as postnatal day (P) 0. Dams were assigned to URB597 or vehicle control groups, and were weighed daily before receiving an i.p. injection of URB597 (1, 3 or 10 mg kg^{-1}) or vehicle of the same volume. URB597 stock was dissolved at 10 mg ml^{-1} in 1:1 ethanol/DMSO and stored at –20°C. Working solutions (1 mg mL^{-1}) were prepared fresh on the day of injection in 18/1/1 saline/cremophor/URB597 stock or a mixture of ethanol and DMSO (1:1) in saline/cremophor for vehicle control (10 μL g^{-1} body weight). Dams were injected daily from E10.5 to E16.5 for mass spectrometry measurements and the embryonic brain development study, and from E10.5 to P7 for behavioural testing. Embryo sex was not determined. On P21, pups were weaned and housed in groups of three to five. Animal procedures were conducted in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010), U.S. Department of Health and Human Services, Baylor College of Medicine and Indiana University guidelines. All procedures were approved by the appropriate institutional animal use and care committees at Baylor and Indiana Universities. Drug and receptor nomenclature adhere to the conventions outlined in Alexander *et al.* (2013).

Tissue processing

Timed-pregnant dams exposed to either URB597 or control vehicle from E10.5–E16.5 were anaesthetized with isoflurane or a single i.p. injection (3 mL kg^{-1}) of a rodent anaesthetic cocktail containing ketamine 37.6 mg mL^{-1}, xylazine 1.92 mg mL^{-1} and acepromazine 0.38 mg mL^{-1} on the 16th day of pregnancy, 3 h after the last injection on E16.5. The embryos were collected by hysterectomy. The embryonic brains were removed and snap frozen for MS, or immersion fixed in 4% paraformaldehyde in PBS overnight at 4°C for immunocytochemistry. Snap-frozen brains were stored at –70°C. The fixed brains were cryoprotected in 30% sucrose and sectioned in the coronal plane with a Leica CM3050S cryostat (Leica Microsystems Nussloch GmbH, Nussloch,

Germany) at 20 µm thickness. The sections were mounted on SuperFrost Plus slides (Fisher Scientific, Hampton, NH, USA) and stored at –20°C until processing. All sections were collected in series of 10, such that each section on the slide was 200 µm apart.

Endocannabinoid quantification

Lipid extracts of tissues were performed as previously described (Bradshaw *et al.*, 2006). Samples were separated using a C18 Zorbax reversed-phase analytical column (Agilent Technologies, Palo Alto, CA, USA). Gradient elution (200 µL min^{-1}) was driven using two Shimadzu 10AdVP pumps (Shimadzu Corp., Kyoto, Japan). Eluted samples were analysed by electrospray ionization using an Applied Biosystems/MDS Sciex (Foster City, CA, USA) API3000 triple quadrupole mass spectrometer. A multiple reaction monitoring (MRM) setting on the LC/MS/MS was then used to analyse levels of each compound present in the sample injection. Synthetic standards were used to generate optimized MRM methods and standard curves for analysis. Results are expressed as % of control levels. Differences were evaluated for significance using the non-parametric Wilcoxon signed rank test.

Immunohistochemistry

Cryostat sections of the brain were immersed in antigen retrieval buffer (0.01 M citric acid, pH 6.0) and microwaved on high setting for 1 min and then on low setting for 10 min, then allowed to cool down to room temperature. Cryosections were washed three times with PBS/0.01% Triton X-100 (PBST) then permeabilized with 0.2% Triton X-100 in PBS at room temperature for 10 min. Non-specific binding was blocked with 3% normal goat serum in PBST for 1 h at room temperature. Sections were then incubated with primary antibodies diluted in PBST/1% normal goat serum/2% BSA at 4°C overnight. The next day, sections were washed with PBST, and incubated at room temperature for 2 h with anti-rat Alexa Fluor 488- or anti-rabbit Cy3-conjugated goat secondary antibodies (each diluted 1/500; Invitrogen, Grand Island, NY, USA). Sections were counterstained with DAPI, washed four times with PBST, and mounted with Vectashield (Vector Labs, Burlingame, CA, USA). All sections in a series were used in the following primary antibody combinations: rabbit anti-Tbr2 (1/1000) and rat anti-Histone H3 (phospho S28) (PH3; 1:200), and rat anti-neural cell adhesion molecule L1 (L1; 1:1000) and rabbit anti-growth associated protein 43 (GAP43; 1:1000).

Imaging

Fluorescent images were taken with a Zeiss AxioImager M1 system (Carl Zeiss, Gottingen, Germany) with 5×/0.16 and 10 × 0.3 Zeiss objectives (magnification/numerical aperture), using AxioVision software (Carl Zeiss). All images were processed in Adobe Photoshop CS2 for brightness/contrast, orientation and background correction to better illustrate the staining patterns.

Analysis of embryonic dorsal telencephalon

For the determination of progenitor and neuronal layer thickness, the area of cortical plate and the entire cortical wall was revealed by DAPI staining. The progenitor layer ventricular (VZ) and subventricular zone (SVZ) was demarcated with Tbr2 staining, which stains SVZ progenitor and intermediate progenitor cells. PH3-positive nuclei were counted 100 µm away from the pallial-subpallial boundary (where the ganglionic eminence starts) in a 400 µm wide strip. The distributions of mitotic apical (cells in VZ) and basal [cells in SVZ and intermediate zone (IZ)] were expressed as % of total PH3-positive nuclei in the field. In similar fields, Tbr2-positive nuclei in the IZ were also counted and their numbers expressed μm^{-2}.

Behavioural testing

Male and female mice at 2–4 months of age were subjected to selected tests from the test battery originally described by Crawley and Paylor (1997). Tests were performed in the order of least stressful to most stressful, with at least 3 days between tests. One cohort of animals was subjected to elevated plus maze and rotarod. Two separate cohorts of animals were used in the following assays: open field, prepulse inhibition (PPI), T-maze, forced swim test and cocaine-conditioned place preference. The age of the animals at the time of each behaviour test was listed in Table 2. Before any behavioural testing, mice were allowed to acclimatize to the testing room for at least 30 min. Behavioural testing was performed between 10:00 and 16:00 (mid phase of light cycle). Experimenters were blind to drug treatment information.

Elevated plus maze

The elevated plus maze was made of four runways (7 × 25 cm) arranged perpendicularly and elevated 40 cm off the ground (Med-Associates, St. Albans, VT, USA). Two arms were enclosed by 15 cm black walls and two arms were open, except for a small 5 mm rim. The test animals were placed in the centre of the elevated maze facing one of the two open arms, and left to explore for 5 min. The number of entries, time spent in the open arms, centre zone or closed arms were manually scored by a trained observer. Arm entries were scored when all four paws were present in the maze arm. Data were analysed using Mann–Whitney *U*-test or two-way (treatment × sex) ANOVA as appropriate.

Open-field assay

Each mouse was placed into the centre of a clear Plexiglas chamber (40 cm x 40 cm x 30 cm) with photo beams to record horizontal and vertical movements of the mouse. Activity was recorded over a 30-min period using a computer-operated VersaMax Animal Activity Monitor System (Accuscan Instruments, Columbus, OH, USA). Testing was performed in the presence of bright overhead lights (~750 lux of illumination) and white noise (55 dB). The centre zone is defined as an unmarked square area (22.5 cm x 25.5 cm) lying in the middle of the arena. Data were collected at 10 min intervals and the following measures were analysed: total distance travelled (cm), vertical activity, time spent in centre zone and the centre to total distance ratio. These ratios were calculated by dividing the centre distance (distance travelled in the arena centre: 22.5 cm x 22.5 cm) by total distance travelled. Data for the 30-min period were analysed using two-way (treatment × sex) ANOVA.

Forced swim test

Each mouse was placed for 6 min in a glass beaker (diameter: 13 cm, height: 19 cm) filled with water (height: 14 cm,

temperature: 22 ± 1°C). Water was changed between mice. The duration of immobility during the last 4 min of a 6-min trial was recorded. Minimal movements made to balance the body and keep the head above the water were scored as immobility. Data were analysed using two-way (treatment × sex) ANOVA.

Rotarod test

Motor coordination and skill learning were tested using an accelerating rotarod (Med-Associates). Mice were placed on a rotating drum (3 cm in diameter), which accelerated at a constant rate from 4 to 40 rpm over a 5-min period. The time spent walking on top of the rod until the mouse either fell off the rod, or slipped and held onto the rod to ride completely around was recorded. Mice were given six trials on 2 consecutive days with a maximum time of 300 s (5 min) per trial and a 60 min inter-trial rest interval. Rotarod data were analysed using a three-way (treatment × sex × trial) ANOVA with repeated measures.

Acoustic startle and PPI of acoustic startle

PPI of the acoustic startle response was measured as described in Paylor and Crawley (1997). Briefly, acoustic startle responses were measured using the SR-Lab startle response system (San Diego Instruments, San Diego, CA, USA). Each mouse was placed in a Plexiglas cylinder within a sound-attenuating chamber and habituated to a 70-dB background white noise for 5 min before the beginning of the test session. Each test session consisted of six blocks, with each block containing eight pseudo-randomized trial types. These included: no stimulus (to measure baseline movement in the cylinder), startle stimulus only (120 dB, 40 ms) and three prepulse stimuli (74, 78, 82 dB; 20 ms) presented either alone or 100 ms before the startle stimulus. The inter-trial intervals ranged from 10 to 20 s. Startle responses, detected as force changes within the Plexiglas cylinder, were recorded every 1 ms during a 65-ms period that followed the onset of either the prepulse during prepulse-alone trials or the startle stimulus. The maximum startle amplitude was used as the dependent variable. Percentage PPI of the startle response was calculated for each prepulse as 100 – [(startle response to trials with prepulse and startle stimulus trials/startle response to trials with startle stimulus alone) × 100]. Acoustic startle response amplitude data were analysed using two-way (treatment × sex) ANOVA. PPI data were analysed using a three-way (treatment × sex × prepulse sound level) ANOVA with repeated measures.

T-maze

The T-maze was made of three runways (5 cm lane width, 35 cm stem length, 28 cm arm length and 10 cm wall height). One arm was perpendicular to two opposed arms. A forced choice paradigm was used: each test block consists of two consecutive trials. At the start of the test, the animal was placed at the base of the 'T', and one forced arm choice was made with one goal arm blocked. After the forced trial, the blockade of the arm was removed, and the animal was allowed to enter either the right or left goal arms (free choice trial). If the arm opposite the one explored in trial 1 is chosen, the mouse has exhibited spontaneous alternation. To increase memory load, there was a 45 s wait time between forced and free choice trials. The blocked arm alternated between each forced trial. Each mouse was tested for three blocks per day and with 3–5 days of rest in between test days. The number of spontaneous alternations made was recorded. If a mouse showed side preference (choosing the same goal arm during free choice trial for three consecutive test blocks), it was eliminated from data analysis (four mice from vehicle group and two mice from URB597 group). Only male mice were used in the test. Data were analysed using Student's *t*-test.

Conditioned place preference

Determination of conditioned place preference for cocaine was performed using a two-chamber conditioned place preference apparatus (Accuscan Instruments). The two sides of the apparatus possessed visually (horizontal white or vertical black stripes on the walls) and tactilely (rough and marbled flooring) distinct environments. Male mice were habituated to the testing room for 2 h before habituation, conditioning and testing trials. Mice were given a 30 min habituation trial where they were allowed to freely explore both sides of the testing chamber (day 1). A pre-testing trial was conducted during which mice were allowed to freely explore both sides of the chamber for 30 min (day 2). The chamber side starting location for the pre- and post-testing sessions was randomly assigned by tossing a coin. Mice showing a pre-conditioning place preference of >65% time spent in either chamber during the pre-testing session were excluded from the experiment (six URB597 and four vehicle mice were excluded for this reason). Eight conditioning trials were conducted on separate days (days 3–10). Mice were divided into two groups, each containing equal numbers of vehicle- and URB597-treated mice. Mice in each group were given either saline or 10 mg kg^{-1} cocaine and placed in one of the two context-dependent sides of the apparatus for 20 min (post-injection). Assignment of conditioning environments was done so that equal numbers of vehicle- and URB597-treated mice were paired with cocaine for each of the two conditioning environments. Each group of mice received cocaine or saline on alternating days so that all mice received a total of four saline and four cocaine conditioning sessions. Saline and cocaine were administered via i.p. injection in a volume of 10 µL g^{-1} body weight. Testing for cocaine-conditioned place preference was performed by placing animals in the testing apparatus for 30 min, with access to both the drug and saline-paired chambers (day 11). The % time spent in the drug-paired side of the testing arena represents the dependent variable for this assay. Conditioned place preference data were first analysed using a two-way (treatment × time) ANOVA, followed by Student's paired *t*-test to compare the reward response (pre- versus post-conditioning) in vehicle- and URB597-exposed animals.

Statistical analyses

Statistical analysis was conducted using SPSS (SPSS, Chicago, IL, USA). All data are presented as mean ± SEM. The level of significance was set at $P \leq 0.05$. The one sample Kolmogorov–Smirnov test was used to test for normality. For data that satisfied the assumption of normality, parametric tests were used. For data that did not meet the assumption of normality, the non-parametric Mann–Whitney *U*-test was used, except

in the case of results from MS where the non-parametric Wilcoxon signed rank test was utilized. Outliers, defined as a value greater or less than twice the SD from the mean, were removed from the data set. One male mouse from URB-treated group was removed from the maximum startle response data set.

Materials

ICR mice were acquired from Charles River (Wilmington, MA, USA). Cocaine was provided by NIDA Drug Supply (Bethesda, MD, USA). Anandamide-d_4 was purchased from Tocris Bioscience (St. Louis, MO, USA). URB597, AEA, *N*-oleoyl ethanolamine, *N*-palmitoyl ethanolamine, *N*-docosahexaenoyl ethanolamine, PGE2, PGF2α and 2-arachidonoyl glycerol were purchased from Cayman Chemical (Ann Arbor, MI, USA). *N*-arachidonoyl glycine was purchased from Biomol (Plymouth Meeting, PA, USA). HPLC-grade water and methanol were purchased from VWR International (Plainview, NY, USA). HPLC-grade acetic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-Tbr2 and rat anti-Histone H3 antibodies were purchased from Abcam (Cambridge, MA, USA). Rat anti-neural cell adhesion molecule L1 and rabbit anti-GAP43 antibodies were from Millipore (Temecula, CA, USA).

Results

General health of dams was not affected by long-term URB597 treatment

To assess the general health of the mice during treatment, dam body weight was monitored daily before drug or vehicle injection from embryonic day (E) 10.5 to postnatal day (P) 7. Litter size at birth and postnatal mortality (the number of pups that died before weaning) were also recorded. No differences were observed in the body weight gains of URB597-treated dams during gestation (Figure 1A) or lactation (Figure 1B) as compared with the vehicle-treated animals (gestation: $F_{1,18}$ = 0.006, P = 0.937; lactation: $F_{1,10}$ = 0.007, P = 0.934; Two-way ANOVA with repeated measure (treatment × day). Body weight steadily increased during pregnancy and lactation in both vehicle- and URB597-treated dams (gestation: $F_{6,108}$ = 310.31, P < 0.001; lactation: $F_{6,60}$ = 38.57, P < 0.001). This suggests that the drug dose used in the present study was not overtly toxic to the dams during gestation and lactation. There were no significant treatment × day interaction effects (gestation: $F_{6,108}$ = 0.693, P = 0.656; lactation: $F_{6,60}$ = 1.335, P = 0.256). Furthermore, perinatal exposure to URB597 did not affect litter size at birth (10.5 ± 1.2 and 12.5 ± 1.3 for vehicle and URB597-treated dams, respectively, n = 6 per group, t_{10} = –1.124, P = 0.287, Student's t-test). There was a non-significant trend towards increased postnatal mortality in pups exposed to URB597 (2.2 ± 0.5 pups per litter) compared with vehicle (1.0 ± 0.4 pups per litter) (6 litters for each treatment group, t_{10} =-1.941, P = 0.081, Student's t-test).

Prenatal URB597 treatment increased embryonic brain levels of anandamide related lipids

AEA and 2-arachidonoyl glycerol (2-AG) are two major endogenous ligands of cannabinoid receptors (Mackiea and Stella, 2006). *N*-stearoylethanolamide (SEA), *N*-palmitoyl ethanolamide (PEA), *N*-oleoylethanolamide (OEA) and *N*-docosahexaenoyl ethanolamide (DHEA) are endogenous fatty acid derivatives that structurally resemble the endocannabinoids. SEA, PEA and OEA have been shown to exert cannabimimetic activity (Watanabe *et al.*, 1999), and DHEA binds to the rat brain CB_1 receptor with a K_i of 324 nM, which is approximately 10-fold higher than the K_i for AEA (Sheskin *et al.*, 1997). *N*-arachidonoyl glycine (NAGly) is an endogenous AEA metabolite and activates GPR18 (Bradshaw *et al.*, 2009; McHugh *et al.*, 2010). PGE2 and PGF2α were recently shown to be metabolites of 2-AG in the brain and inhibition of 2-AG metabolism strongly affects their levels (Nomura *et al.*, 2011).

To explore whether maternal treatment with URB597 affects the levels of endogenous cannabinoids and related

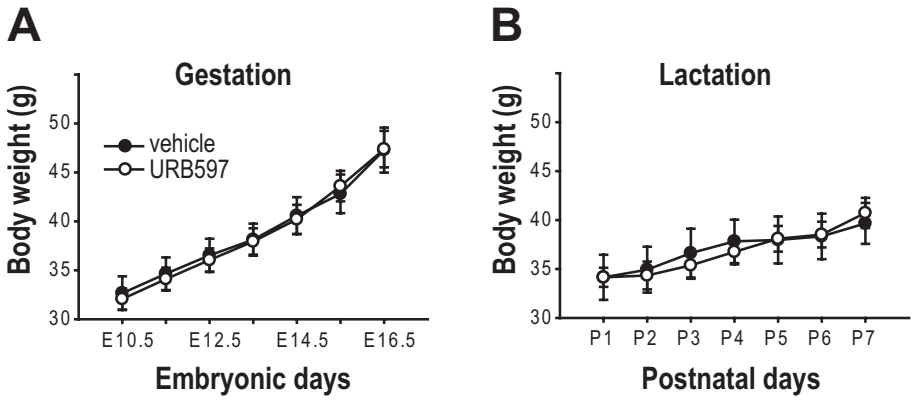

Figure 1

Normal body weight gain of the dams exposed to URB597 during gestation and lactation. There were no significant alterations in body weight of the dams exposed to URB597 during gestation (A) or lactation (B), compared to the vehicle-treated group. Body weights were measured daily, before drug administration.

Table 1

URB597 increases embryonic acyl amide levels

Lipid	1 mg kg^{-1} URB597	*P* value	3 mg kg^{-1} URB597	*P* value	10 mg kg^{-1} URB597	*P* value
AEA	157 ± 9	0.001	179 ± 32	0.033	207 ± 21	<0.001
OEA	167 ± 5	<0.001	397 ± 80	0.036	439 ± 38	<0.001
PEA	173 ± 12	0.028	394 ± 78	0.036	458 ± 40	0.005
SEA	187 ± 9	<0.001	312 ± 62	0.008	452 ± 49	<0.001
DHEA	152 ± 9	0.002	306 ± 56	0.036	356 ± 35	<0.001

Values are listed as mean % of baseline± SEM. Abbreviations used with baseline levels in parentheses. AEA, *N*-arachidonoyl ethanolamide (1.31 ± 0.15 pmol g^{-1}); DHEA, *N*-docosahexaenoyl ethanolamide (11.7 ± 0.6 pmol g^{-1}); OEA, *N*-oleoylethanolamide (35.2 ± 9.5 pmol g^{-1}); PEA, *N*-palmitoylethanolamide (24.2 ± 8.82 pmol g^{-1}); SEA, *N*-stearoylethanolamide (41.1 ± 9.3 pmol g^{-1}). Data were summarized from 6, 8 or 10 embryos treated with 1, 3 or 10 mg kg^{-1} URB597, respectively, and normalized to the values from 17 vehicle-treated embryos. *P* values are versus vehicle levels.

lipids in the developing embryos, lipid extracts of brains of vehicle- and URB597-exposed embryos were prepared for mass spectrometric analysis. AEA levels were significantly increased in the URB597-exposed embryos, demonstrating that maternal FAAH inhibitors increase embryonic brain AEA. Increasing doses of URB597 also progressively and significantly elevated the levels of related *N*-acyl amides, including OEA, PEA, DHEA and SEA (Table 1). In contrast, levels of the endocannabinoid 2-AG, the prostaglandins, PGE2 and PGF2α, and NAGly (data not shown) were similar in vehicle- and 10 mg kg^{-1} URB597-exposed embryonic brains. In summary, perinatal exposure to URB597 significantly increased AEA and related FAAH substrates, while 2-AG and several other related signalling lipids were unchanged.

Perinatal URB597 did not affect several anatomical measures of embryonic brain development

To assess the effect of an efficacious, but not-overtly toxic dose of URB597 on brain development, we compared the pattern of axonal tracts and mitosis of neuroprogenitor cells in URB597- and vehicle-exposed embryonic brains. We have previously shown that knockout or blockade of CB_1 receptors during embryonic development leads to abnormal axonal tract patterns, with enlarged axon fasciculation and axon misrouting, most prominent in the pallial-subpallial boundary; while alterations in neuronal proliferation and migration were found in CB_1 receptor and FAAH knockout embryos (Mulder *et al.*, 2008; Wu *et al.*, 2010). Here, we compared the cortical and subcortical axonal tract patterns in URB597- and vehicle-exposed embryos at E16.5, using the axonal tract markers Neural Cell Adhesion Molecule L1 (L1) and GAP43. Embryos were exposed to URB597 or vehicle control from E10.5 to E16.5, through daily drug administration to dams. Both URB597- and vehicle-exposed embryonic brains showed typical thalamocortical and corticothalamic/corticofugal tract patterns, with multiple fascicles in the striatum, turning at the pallial-subpallial boundary, and continuing to navigate towards the cortical plate or specific subcortical targets respectively (Figure 2A–B). No abnormal fasciculation or misrouting of axons was apparent in URB597-exposed embryonic brains.

We then investigated whether prenatal URB597 exposure affects progenitor cell cycle progression and division, using the mitosis marker PH3 (De Pietri Tonelli *et al.*, 2008). Immunostaining for PH3 showed that the location and pattern of mitotic apical (VZ) and basal (SVZ + IZ) progenitors were similar in URB597- and vehicle-exposed E16.5 dorsal telencephalon (Figure 2C–D). Tbr2 staining was used to identify intermediate progenitor cells that are normally present in the subventricular zone (Englund *et al.*, 2005). A total of 23 sections from six brains from three independent litters were analysed for vehicle-exposed group, and 14 sections from four brains from two independent litters were analysed for the URB597-exposed group. Quantification of PH3-positive nuclei in the VZ and SVZ/IZ showed no significant difference in the distribution of apical and basal progenitors undergoing mitosis in URB597- and vehicle-exposed embryos (Figure 2E; apical progenitors: URB597-exposed brains = 59.4 ± 3.2 %, vehicle-exposed brains = 61.9 ± 2.4 %; t_{35} = 0.640, P = 0.526; basal progenitors: URB597-exposed brains = 39.7 ± 3.1 %, vehicle-exposed brains = 37.5 ± 2.4 %; t_{35} = –0.578, P = 0.567, Student's *t*-test).

Further morphological analysis showed that cortical thickness in URB597- and vehicle-exposed embryos was similar (Figure 2F; URB597-exposed brains = 661.0 ± 16.7 μm, vehicle-exposed brains = 645.3 ± 10.9 μm; t_{35} = –0.822. P = 0.417, Student's *t*-test). There was also no change in Tbr2-positive nuclei in the intermediate zone in the URB597-exposed brains (Figure 2G; URB-597 exposed brains = 17.0 ± 0.9 nuclei mm^{-2}, mean rank = 11.0, vehicle-exposed brains = 21.6 ± 3.6 nuclei mm^{-2}, mean rank = 14.1; U = 54, P = 0.329, Mann–Whitney *U*-test). Taken together, these results suggest that prenatal exposure to URB597 from E10.5 to E16.5 does not affect axonal development or progenitor cell cycle progression and division.

A panel of behavioural assays reveals that perinatal URB597 leads to several subtle behavioural consequences

Reproductive and MS data showed that 10 mg kg^{-1} URB597, while effective at increasing brain levels of AEA and related *N*-acyl amides, was non-toxic to the dams during gestation

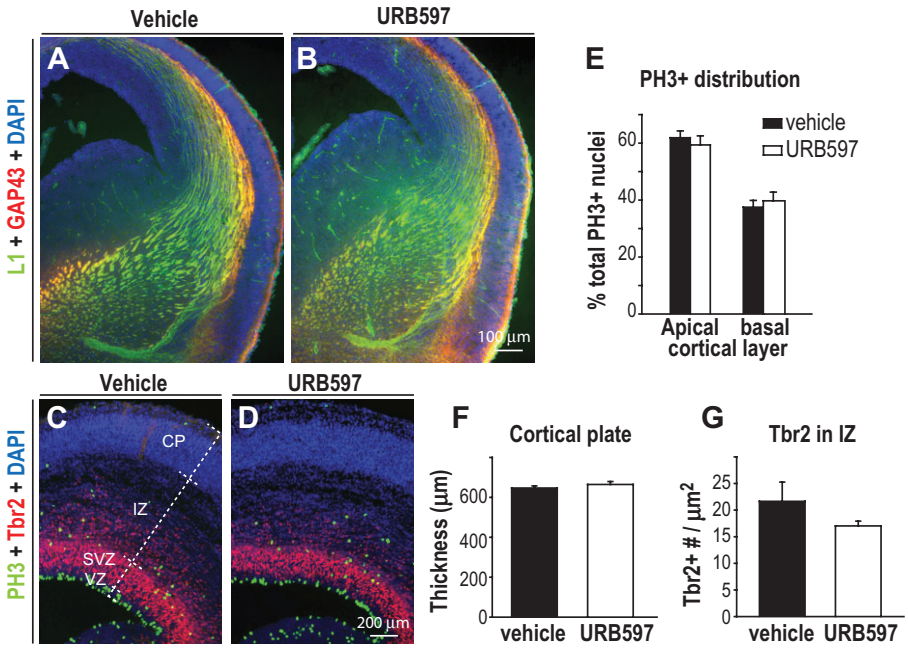

Figure 2

Normal axonal development and progenitor cell cycle progression and division in embryos exposed to URB597. (A–B) Merged images showing the pattern of L1-(green) and GAP43-(red) axonal tracts in URB597- and vehicle-exposed embryonic brains at E16.5. URB597-exposed brains showed normal axonal tracts and fasciculation patterns compared to controls. (C–D) Merged images showed PH3-labelled mitotic progenitor cells (green) and Tbr2-labelled intermediate progenitor cells (red) in URB597- and vehicle-exposed brains at E16.5. Sections were counterstained with DAPI (blue). (E) Quantification of apical (ventricular zone) and basal (subventricular and intermediate zone) PH3-positive progenitor cells, expressed as percentage of total PH3-positive nuclei. The distribution of apical and basal progenitors in URB597-exposed embryos was similar to vehicle-exposed embryos. (F) Cortical thickness, measured 200 µm away from the end of the pallial-subpallial boundary (dashed line), was similar between URB597- and vehicle-exposed embryos at E16.5. (G) Quantification of Tbr2-positive intermediate progenitor cells in the intermediate zone showed that the number of progenitors was similar in URB597- and vehicle-exposed brains. Data were analysed using Student's *t*-test. Cp, cortical plate; iz, intermediate zone; svz, subventricular zone; vz, ventricular zone.

and lactation and did not alter the measures of neurodevelopment that were investigated. Thus, we next examined the effects of perinatal (E10.5 to P7) treatment with this dose of URB597 on selected adult behaviours using a battery of behavioural tests.

Anxiety and spontaneous activity were normal in URB597-exposed offspring

To explore the effect of perinatal exposure to URB597 on anxiety levels in mice later in life, we performed the elevated plus maze and open field tests, well-validated assays used to assess anxiety in rodents (Crawley and Paylor, 1997). Greater than 50% of URB597-exposed animals (9 out of 17) did not enter the open arm during the examination period while only a few vehicle-exposed animals avoided the open arm (3 out of 18) (Figure 3). However, these differences between vehicle and URB-treated animals were not statistically significant, either for the percentage of time spent in the open arm (Mann–Whitney *U*-test: males, $U = 32.0$, $P = 0.690$, $r = 0.10$; females, $U = 21.0$, $P = 0.082$, $r = 0.41$) or the number of open arm entries (Mann–Whitney *U*-test: males, $U = 26.5$, $P = 0.343$, $r = 0.23$; females, $U = 19.5$, $P = 0.060$, $r = 0.44$) (Figure 3A–B; Table 2). There was no overall difference between the treatment or sex in the % time spent in closed arms (treatment: $F_{1,\ 31} = 2.39$, $P = 0.132$; sex: $F_{1,\ 31} = 3.98$, $P = 0.055$) or in the number of closed arm entries (treatment: $F_{1,\ 31} = 1.88$, $P = 0.180$; sex: $F_{1,\ 31} = 0.28$, $P = 0.601$) (Figure 3C–D; Table 2). Furthermore, no significant treatment and sex interaction effect were found (% closed arm time: $F_{1,\ 31} = 1.29$, $P = 0.265$; for closed arm entries: $F_{1,31} = 1.40$, $P = 0.246$). URB597-exposed mice tended to have less overall activity as measured by total arm entries ($F_{1,31} = 3.58$, $P = 0.068$), although this did not reach statistical significance. There was no significant sex or treatment × sex interaction effect for total arm entries (sex: $F_{1,31} = 0.004$, $P = 0.952$; treatment x sex: $F_{1,31} = 0.18$, $P = 0.672$).

The lack of a definitive anxiety phenotype in the adult offspring of dams that had been exposed to URB597 was confirmed in a separate cohort of animals, using the open field assay (Figure 4). In the open field test, each mouse was placed in the centre of the open field arena and left to explore for 30 min. The sum of total distance travelled, vertical activity, time spent and distance travelled in the centre zone, and centre distance ratio for the 30-min test period is shown in Table 2. In the open field assay, the ratio of centre distance to total distance travelled (to normalize for activity levels) and time spent in the centre zone provide measures of the anxiety-related responses to a bright and open arena (Peier *et al.*, 2000; Tritto *et al.*, 2004). Adult mice perinatally

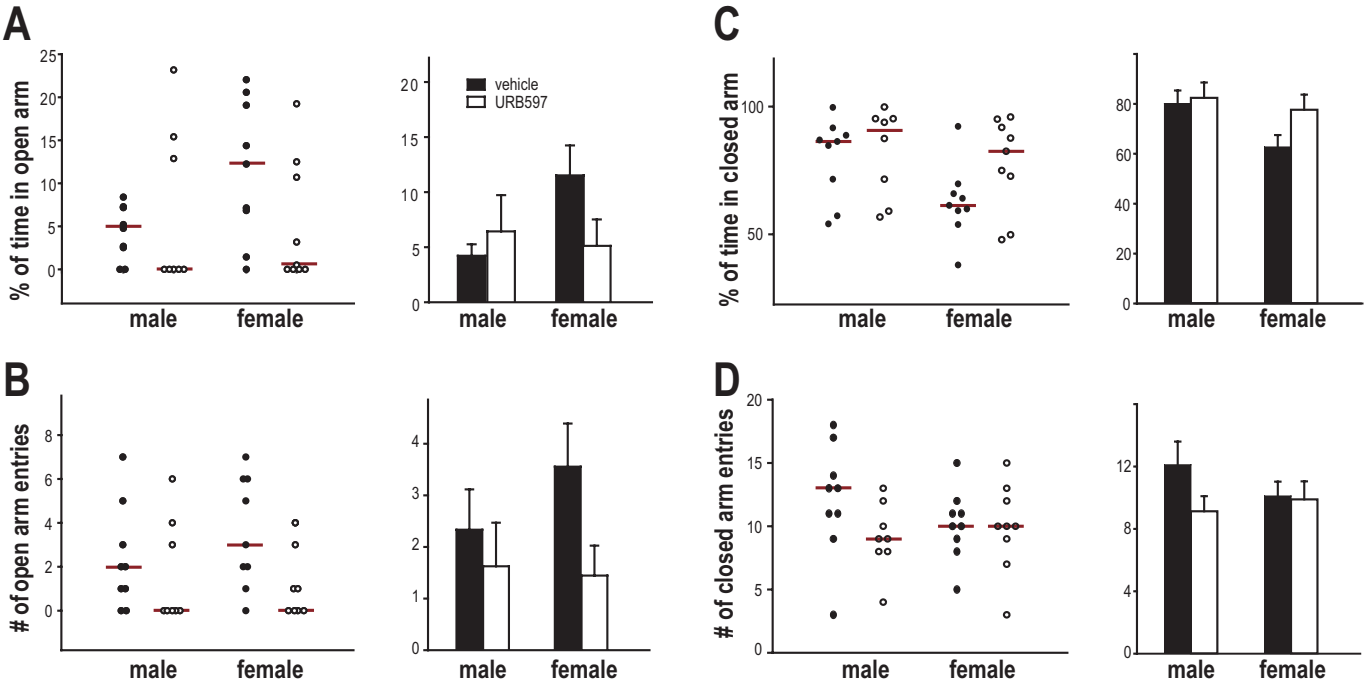

Figure 3

URB597-exposed adult offspring showed normal levels of anxiety. Anxiety-like behaviours were examined by elevated plus maze at 10–12 weeks of age. No statistically significant difference was found for the % of time spent in the open arm (A), the number of entries into the open arms (B), the % of time spent in the close arm (C), or the number of entries into the close arm (D) between either male or female URB597-exposed adult offspring and vehicle-exposed controls. The values for the open arm entries and % of time violated homogeneity of variance (tested with Levene's test of equality of error variances). Values from individual animals are shown in the distribution graph (with medians indicated), while mean ± SEM are presented as bar graphs.

exposed to URB597 were indistinguishable from vehicle-treated controls in both time spent in centre zone (Figure 4C; treatment: $F_{1,53} = 1.303$, $P = 0.259$, sex: $F_{1,53} = 0.047$, $P = 0.830$) or centre to total distance ratio (Figure 4D; treatment: $F_{1,53} = 1.540$, $P = 0.220$, sex: $F_{1,53} = 0.992$, $P = 0.324$; Table 2). There was no significant interaction effect for either parameter (centre distance ratio: $F_{1,53} = 0.229$, $P = 0.634$; centre zone time: $F_{1,53} = 0.295$, $P = 0.590$). Taken together with the data from elevated plus maze, perinatal exposure to URB597 did not significantly affect anxiety levels in adult offspring.

The open field paradigm also allows for simultaneous assessment of spontaneous exploratory activity in addition to assessing anxiety levels (Crawley and Paylor, 1997). There was no overall treatment or sex effect on horizontal activity in the open field arena, measured by total distance travelled (Figure 4A; treatment: $F_{1,53} = 0.021$, $P = 0.884$, sex: $F_{1,53} = 0.885$, $P = 0.351$). There was no treatment or sex effect on vertical activity either (Figure 4B; treatment: $F_{1,53} = 0.409$, $P = 0.525$, sex: $F_{1,53} = 0.511$, $P = 0.478$). There was also no significant interaction effect for total distance travelled ($F_{1,53} = 2.212$, $P = 0.143$) or vertical activity ($F_{1,53} = 0.003$, $P = 0.955$).

Motor coordination and learning are normal in URB597-exposed offspring

We assessed motor coordination/control and balance in vehicle- and URB597-exposed adult offspring on the accelerating rotarod (Figure 4E). There were no significant differences between the treatments ($F_{1,28} = 0.209$, $P = 0.651$), sex ($F_{1,28} = 0.131$, $P = 0.720$), and no genotype × sex interaction effects ($F_{1,28} = 0.998$, $P = 0.326$). All mice improved significantly during training ($F_{5,140} = 9.607$, $P < 0.001$), indicating that mice perinatally exposed to URB597 were able to acquire coordinated motor behaviour as well as the vehicle control mice, since the time they stayed on a rotating rod increased significantly during repeated trials. There was no significant treatment × trial ($F_{5,140} = 0.843$, $P = 0.522$), sex × trial ($F_{5,140} = 0.920$, $P = 0.470$) or treatment × sex × trial ($F_{5,140} = 1.369$, $P = 0.239$) interaction effects.

Normal sensorimotor gating in URB597-exposed offspring

PPI of the acoustic startle reflex (PPI) is a phenomenon in which a weak non-startling sound (pre-stimulus) suppresses the startle response to a strong acoustic startle stimulus presented immediately after the pre-stimulus. As the prepulse level increases, the suppression of the startle response also increases. PPI provides an operational measure of sensorimotor gating processes in humans and mice (Geyer *et al.*, 2002).

Overall, there were no significant effects of treatment and sex on the maximum response at 120 dB (Figure 4F; treatment:$F_{1,50} = 1.608$, $P = 0.211$, sex: $F_{1,50} = 0.180$, $P = 0.673$).

Table 2

Summary of behavioural data

Behavioural paradigm (age in months)	Measurement	Male		Female		Treatment effect	Sex effect
		VEH	URB	VEH	URB	*P* value	*P* value
Body weights	weight (g)	27.8 ± 0.8 (12)	27.2 ± 0.4 (19)	20.8 ± 0.4 (15)	21.7 ± 0.5 (17)	0.747	<0.001
Elevated plus maze (3–3.5)	% of time spent in open arm	4.22 ± 1.04 (9)	6.43 ± 3.30 (8)	11.52 ± 2.72 (9)	5.12 ± 2.40 (9)	0.401	0.232
	# of open arm entries	2.33 ± 0.78 (9)	1.63 ± 0.84 (8)	3.56 ± 0.84 (9)	1.44 ± 0.58 (9)	0.075	0.501
	% of time spent in closed arm	80.14 ± 5.23 (9)	82.42 ± 6.14 (8)	62.73 ± 4.78 (9)	77.64 ± 6.07 (9)	0.132	0.055
	# of closed arm entries	12.11 ± 1.49 (9)	9.13 ± 0.97 (8)	10.11 ± 0.92 (9)	9.89 ± 1.16 (9)	0.180	0.601
Open field (3–3.5)	total distance travelled (m)	54.69 ± 9.28 (9)	47.26 ± 3.91 (16)	51.66 ± 4.15 (15)	60.71 ± 5.51 (17)	0.884	0.351
	vertical activity (# beam breaks)	543.78 ± 91.48 (9)	596.69 ± 47.34 (16)	602.40 ± 75.31 (15)	646.71 ± 80.44 (17)	0.525	0.478
	time in centre zone (s)	198.61 ± 21.91 (9)	242.19 ± 28.73 (16)	218.24 ± 23.99 (15)	233.72 ± 21.08 (17)	0.259	0.830
	ratios of centre to total distance	0.25 ± 0.02 (9)	0.27 ± 0.01 (16)	0.26 ± 0.02 (15)	0.24 ± 0.02 (17)	0.220	0.324
Forced swim test (3)	normalized immobility (% of control)	100.00 ± 8.54 (22)	121.05 ± 5.53 (15)	100.00 ± 15.40 (8)	118.47 ± 3.68 (10)	0.042	0.899
Prepulse inhibition (3–3.5)	% of PPI at 74 dB prepulse	7.46 ± 7.48 (8)	22.12 ± 8.29 (16)	13.95 ± 9.53 (15)	18.71 ± 10.66 (16)	0.342	0.880
	% of PPI at 78 dB prepulse	21.90 ± 5.48 (8)	45.75 ± 5.73 (16)	26.92 ± 15.31 (15)	37.83 ± 8.68 (16)	0.118	0.895
	% PPI at 82 dB prepulse	43.58 ± 11.53 (8)	59.43 ± 7.00 (16)	47.59 ± 13.86 (15)	66.57 ± 5.76 (16)	0.095	0.588
	Startle response (A.U.)	513.79 ± 123.93 (8)	906.52 ± 154.06 (15)	684.12 ± 118.98 (15)	624.75 ± 90.16 (16)	0.211	0.673
T-maze (3–4)	% alternation	62.13 ± 3.40 (18)	49.09 ± 3.03 (17)	N.A.	N.A.	0.007	N.A.

Values are listed as mean ± SEM (animal #). A.U., artificial unit; VEH, vehicle-treated group; URB, URB597-treated group.

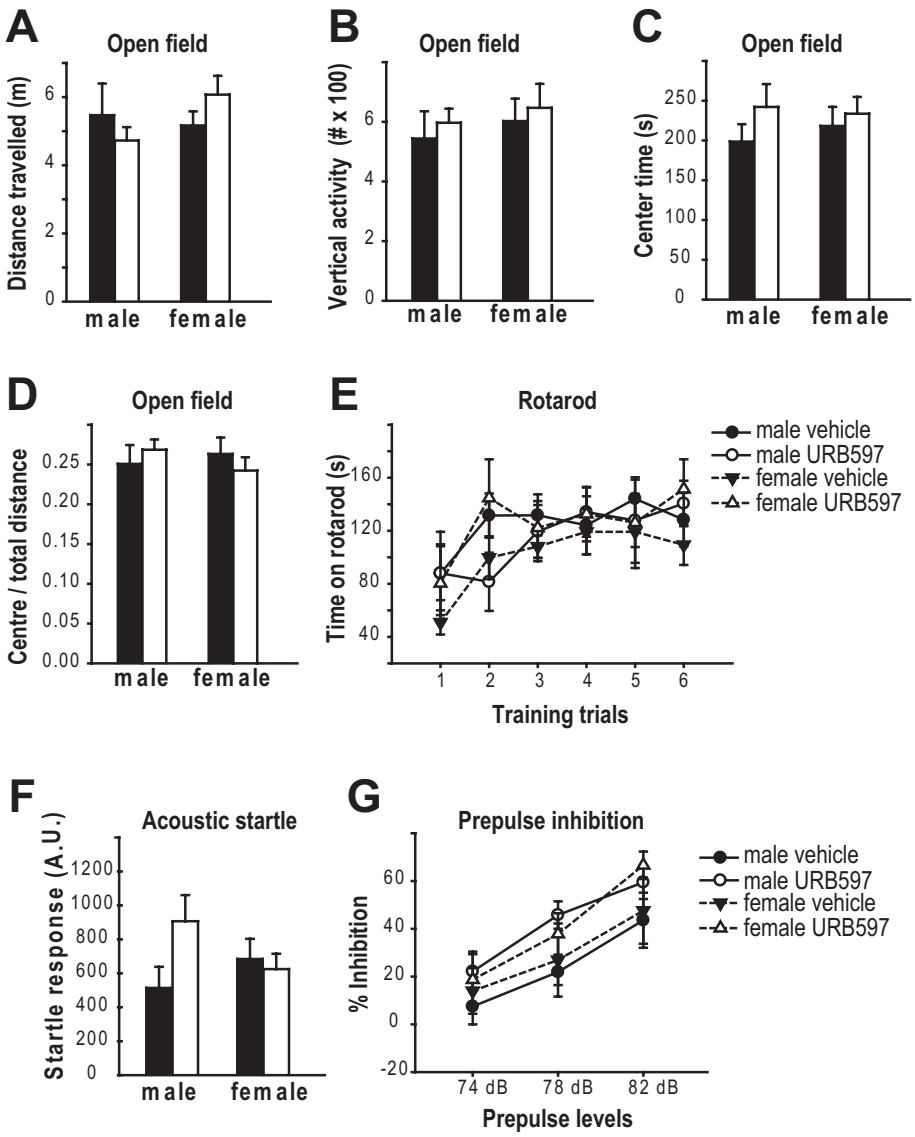

Figure 4

URB597-exposed adult offspring showed normal locomotion, motor function, and sensory-motor gating. (A–D) Spontaneous activity measured in the open field assay. Total distance travelled for 30 min in a novel environment (A) and vertical activities (B) were similar between male and female URB597- and vehicle-exposed adult offspring. Anxiety levels as measured by time spent in centre zone (C) and centre distance ratio (D) were similar between male and female URB597- and vehicle-exposed adult offspring. (E) Motor skill learning and coordination were assessed in the accelerating rotarod test. Time spent walking on top of the rotarod during six trials is shown. There was no significant difference in motor performance between male or female URB597- or vehicle-exposed adult offspring. (F, G) Sensorimotor gating was measured by prepulse inhibition (PPI) of the acoustic startle response. (F) Maximum startle response to 120 dB white noise sound burst is shown. Male and female URB597-exposed adult offspring showed similar startle responses compared to vehicle-exposed controls. (G) Inhibition of the acoustic startle response was determined with three prepulse levels (74, 78 and 82 dB). Male and female URB597-exposed adult offspring showed similar PPI compared with vehicle-exposed controls at all prepulse levels tested. Values are shown in Table 2. Data were analysed using two-way ANOVA (treatment × sex), except for rotarod and PPI data, where three-way ANOVA with repeated measures were used.

There was no treatment × sex interaction effect ($F_{1,\ 50} = 2.958$, $P = 0.092$). For PPI, there was a main effect of prepulse level ($F_{2,\ 102} = 36.839$, $P < 0.001$) as expected (Figure 4G). Thus, as the prepulse level increased, there was greater suppression of the startle response. We found no significant difference in the percentages of PPI between adult offspring perinatally exposed to vehicle or URB597 (treatment: $F_{1,\ 51} = 2.698$, $P = 0.107$, sex: $F_{1,\ 51} = 0.044$, $P = 0.835$). There was no sex × prepulse level ($F_{2,\ 102} = 0.305$, $P = 0.738$), treatment × prepulse level ($F_{2,\ 102} = 0.483$, $P = 0.618$), treatment × sex × prepulse level ($F_{2,\ 102} = 0.446$, $P = 0.641$) or treatment × sex ($F_{1,\ 51} = 0.132$, $P = 0.718$) interaction effects. In summary, adult offspring perinatally exposed to URB597 exhibit normal sensory-motor integration.

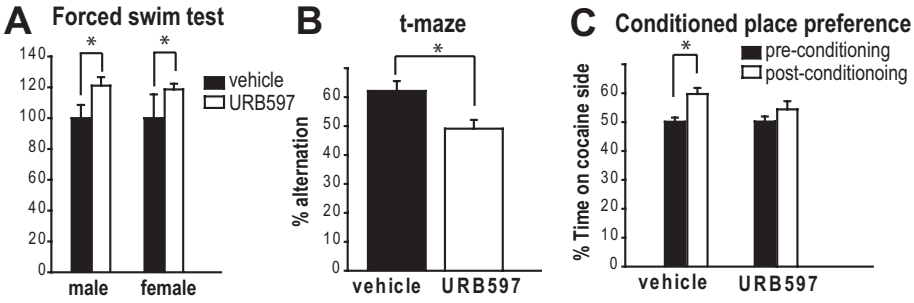

Figure 5

URB597-exposed adult offspring exhibit increased depressive behaviour, impaired working memory and reduced cocaine reward. (A) Depressive behaviour was assessed in the forced swim test. Time spent floating in water (immobile) as percentage of control was shown. URB597-exposed adult offspring spent significantly more time being immobile suggesting increased depressive behaviour (increased helplessness). (B) Working memory was assessed in the t-maze test, using the forced choice paradigm. Only male mice were tested. URB597-exposed adult offspring showed significantly reduced spontaneous alternations (close to chance), suggesting impaired working memory. (C) Reward behaviour was assessed in the conditioned place preference test. Vehicle-exposed adult offspring showed significantly increased preference for the cocaine-paired side, as expected. This preference was abolished in URB597-exposed adult offspring. Data were analysed with two-way ANOVA (treatment × sex) for (A), and with Student's *t*-test for (B) and (C).

Increased depressive behaviour in URB597-exposed offspring

Cannabinoids are also known to influence depressive behaviours (Mangieri and Piomelli, 2007). Depressive behaviour was assessed in adults that had been perinatally exposed to vehicle or URB597 using the forced swim test, one of the most widely used tools for screening antidepressants (Petit-Demouliere *et al.*, 2005). There was a trend for increased immobility time in both male and female mice that had been previously exposed to URB597, albeit with no statistically significant difference (treatment: $F_{1,30}$ = 3.482, P = 0.072, sex: $F_{1,30}$ = 0.049, P = 0.826; treatment × sex interaction effect: $F_{1,30}$ = 0.049, P = 0.826). This data set was generated in the facility at Indiana University. A similar trend for increased immobility time for URB597-exposed male mice was found in a separate cohort of animals prepared and tested in the facility at Baylor College of Medicine by a different trained observer. Comparison between the male control mice showed no statistically significant difference ($t_{17.8}$ = 0.000, P = 1.000). Hence, data were pooled from the two cohorts. When the data were combined, adult offspring perinatally exposed to URB597 showed a slight but significant increase in immobility time in the forced swim test (treatment: $F_{1,51}$ = 4.357, P = 0.042; sex: $F_{1,51}$ = 0.016, P = 0.899; treatment × sex: $F_{1,51}$ = 0.016, P = 0.899; Table 2, Figure 5A). This suggests that perinatal exposure to URB597 increases depressive behaviours later in life. This effect is subtle since a large sample size was required to reach statistical significance. Nevertheless, the fact that the final data set was generated from two separate cohorts of animals and with experiments conducted by different experimenters, at two different facilities lends validity to this observation.

Impaired working memory in URB597-exposed offspring

To explore the effect of perinatal exposure to URB597 on learning and memory later in life, we employed the spontaneous T-maze alternation task to assess spatial learning and alternation behaviour. The T-maze is a 'foraging' task, and the natural tendency of rodents in a T-maze is to alternate their choice of goal arm (Deacon and Rawlins, 2006). Alternation reflects the motivation of the animals to explore their environment, and the response on each trial varies according to what they have previously just done, i.e. using 'working memory'. Moreover, hippocampal dysfunction involving deletion of the GluA1 (also known as GluR-A) AMPA receptor subunit was only detected by T-maze alternation and not by using the Morris water maze, suggesting that this technique is a very sensitive indicator of hippocampal dysfunction (Reisel *et al.*, 2002). To reduce variability associated with the oestrus cycle, only male mice perinatally exposed to vehicle or URB597 were tested. We found that URB597-exposed offspring showed a slight but statistically significant decrease in alternation behaviour, suggesting impairment of working memory (t_{33}=2.854, P = 0.007) (Figure 5B).

Cocaine-conditioned place preference is abolished in URB597-exposed offspring

It has been hypothesized that previous exposure to *Cannabis* increases the likelihood of becoming addicted to other drugs. Interestingly, rats previously exposed to Δ9-tetrahydrocannabinol (Δ^9-THC) showed increased heroin self-administration under continuous reinforcement (with a fixed-ratio schedule) (Solinas *et al.*, 2005). In contrast, rats previously exposed to Δ^9-THC showed no change in cocaine-seeking behaviour under the fixed-ratio schedule, but showed significantly reduced cocaine-seeking behaviour under a progressive-ratio schedule of drug administration (i.e. when the effort required to self-administer drug is high) (Panlilio *et al.*, 2007). To see whether perinatal exposure to URB597 affects reward behaviour later in life, we employed the conditioned place preference paradigm. Conditioned place preference paradigm is a standard behavioural model used to study the rewarding and aversive effects of drugs. The basic characteristics of this assay involve the association of a particular environment with drug treatment (e.g. cocaine),

followed by the association of a different environment with the absence of the drug (i.e. the drug's vehicle). Conditioned place preference occurs if animals spend significantly more time in the drug-paired compartment versus the vehicle-paired compartment when given free access to both compartments in the absence of drug (Prus *et al.*, 2009).

Adult offspring perinatally exposed to URB597 or vehicle were tested for conditioned place preference for 10 mg kg^{-1} cocaine. ANOVA (treatment × time) analysis of the data showed that, as expected, conditioning (time) had a significant effect ($F_{1,\ 100}$ = 11.337, P = 0.001) on the % time spent in the cocaine-paired compartment. There was no significant effect ($F_{1,\ 100}$ = 1.647, P = 0.202) of treatment alone (comparison of combined pre- and post-tests between treatment conditions). However, Bonferroni *post hoc* tests revealed a significant conditioned place preference for vehicle ($F_{1,\ 100}$ = 3.595, P < 0.001) but not URB597-treated mice ($F_{1,\ 100}$ = 1.354, P < 0.001) following conditioning. ANOVA detected no significant treatment × time interaction effect ($F_{1,\ 100}$ = 1.716, P = 0.193). The results of *post hoc* testing were confirmed by data analysis using Student's paired *t*-test. These analyses show that mice perinatally exposed to vehicle spent significantly more time in the drug-associated environment post-conditioning, as indicated by increased preference ratio (t_{58} =-3.784, P < 0.001; paired *t*-test) (Figure 5C). In contrast, this increase in preference ratio after cocaine conditioning was absent in mice perinatally exposed to URB597 (t_{42} =-1.271, P = 0.211). In summary, these data suggest that the regimen of perinatal exposure to URB597 used in the present study decreased cocaine reward.

Discussion

FAAH inhibitors show therapeutic promise in preclinical models of chronic pain, depression and anxiety (Bortolato *et al.*, 2007; Sciolino *et al.*, 2011; Guindon *et al.*, 2013), but also see (Naidu *et al.*, 2007). Endogenous cannabinoids, including AEA, have been implicated in neurodevelopment (Campolongo *et al.*, 2011; Keimpema *et al.*, 2011; Wu *et al.*, 2011; Diaz-Alonso *et al.*, 2012), but a systematic study on the effects of perinatal manipulation of eCBs on subsequent adult behaviours has not been conducted. We undertook this study to examine if FAAH inhibitors had measurable effects on neurodevelopment or later behaviours in the adult using mice as a model organism.

In our initial study, we determined whether doses ranging from 1 to 10 mg kg^{-1} of the well-characterized FAAH inhibitor, URB597, increased embryonic anandamide levels and affected several cannabinoid-sensitive aspects of embryonic cortical development by treating mice from E10.5-E16.5. These doses of URB597 progressively and robustly increased embryonic anandamide levels by about twofold (Table 1), indicating that maternal URB597 effectively increases fetal acyl amides. This is probably due to inhibition of fetal FAAH as URB597 at 1 mg kg^{-1} i.p. inhibits FAAH activity in the rodent fetus and placenta (Daniele Piomelli, personal communication). In addition to degrading anandamide, FAAH is required for the degradation of a number of N-acyl amides, many of which, while biologically active, do not engage cannabinoid receptors (Saghatelian *et al.*, 2006). As expected, other FAAH substrates, including the *N*-acyl amides, OEA, PEA, SEA and DHEA were increased by three- to fourfold (Table 1). In contrast, levels of 2-AG, NAGly and the prostaglandins, PGE2 and PGF2α were unchanged by URB597 treatment. Since FAAH inhibition increases the levels of a range of bioactive lipids, it is important to keep in mind that the consequences of perinatal FAAH inhibition may be mediated by those lipids acting through other targets, including PPARα, TRPV1, GPR18 and GPR119 (Zygmunt *et al.*, 1999; Lo Verme *et al.*, 2005; Kohno *et al.*, 2006; Overton *et al.*, 2006).

An earlier study reported a pro-proliferative phenotype of FAAH knockout in embryonic brains (Aguado *et al.*, 2005). Our results examining proliferation in E16.5 embryos found no evidence for increased proliferation following URB597 treatment (Figure 2). One reason for the difference between the earlier and current studies could be the complete lack of FAAH throughout development in the FAAH knockouts. In addition, prenatal URB597 treatment with 10 mg kg^{-1} only increased AEA levels twofold, compared to the FAAH knockout, where anandamide levels are typically increased ~15-fold (Cravatt *et al.*, 2001). It is possible that increased neural progenitor proliferation is only seen at the exceedingly high AEA (and/or other acyl amide) levels that occur in FAAH knockout mice, and are not observed when acyl amides are more modestly increased, as might occur during therapeutic inhibition of FAAH.

Despite examining a range of behavioural tests, perinatal FAAH inhibition had relatively modest effects. Measures of anxiety, sensorimotor integration and motor learning were not significantly different between URB597 and vehicle-treated mice (Figures 3,4). These results suggest that perinatal FAAH inhibition does not have lasting effects on neural circuits underlying these rather disparate types of behaviour.

Perinatal FAAH inhibition did affect measures of depressive behaviour, working memory and reward in adult mice. The size of the effect on immobility in the forced swim test was modest. It would be interesting to follow-up this finding and determine if other measures of learned helplessness, such as tail suspension, or other measures of depressive behaviours (e.g. sucrose preference and novelty-suppressed feeding) are also abnormal in URB597-treated offspring. URB597 treatment led to decreased alternation in the T-maze task. This suggests that these mice may have impaired working memory. Finally, the blunted conditioned place preference for cocaine in the URB597-treated mice suggests that perinatal FAAH inhibition may alter the proper development of reward circuits. It will be very interesting to see if this effect is also present for other (e.g. natural and other drugs of abuse) forms of reward.

The prenatal and early postnatal periods present a time when the developing brain is most susceptible to teratogens such as infectious agents, drugs and substances of abuse (Diav-Citrin, 2011; Minnes *et al.*, 2011). Clinical studies suggest that maternal *Cannabis* exposure leads to negative effects on impulsivity, executive function and to increased likelihood of early tobacco and *Cannabis* use later in life (Minnes *et al.*, 2011; Wu *et al.*, 2011). These data are paralleled in rodent models where prenatal cannabinoid exposure leads to cognitive impairments, altered emotional behaviour and enhanced sensitivity to drugs of abuse in the adult offspring (Campolongo *et al.*, 2007; 2011; Trezza *et al.*, 2008;

Viveros *et al.*, 2012). Oral administration of Δ^9-THC, the active ingredient of *Cannabis*, from gestational day 15 to postnatal day 9 at a dose that is not associated with gross malformations and/or overt signs of toxicity, induced a long-term memory impairment in the adult rat offspring, as revealed by the inhibitory avoidance test, and also caused a disruption in short-term olfactory memory, as assessed in the social discrimination test (Campolongo *et al.*, 2007). With the same dosing regimen, a long-lasting effect of perinatal Δ^9-THC exposure on emotional reactivity has also been reported (Trezza *et al.*, 2008). In addition, maternal Δ^9-THC exposure has been found to induce sensitization to opiates in morphine- (Rubio *et al.*, 1998) or heroin-induced (Singh *et al.*, 2006) place conditioning in the adult rat offspring. In our studies, we found that a modest elevation of AEA and related acyl amides in the perinatal period impaired working memory and mildly increased depressive behaviours, while it did not affect anxiety, sensorimotor integration and motor function. In contrast to increased sensitization to opiates in adult rat offspring perinatally exposed to Δ^9-THC, our study showed reduced cocaine-conditioned preference in adult mouse offspring perinatally exposed to FAAH inhibitors. Together, these results suggest that perinatal inhibition of FAAH produces much more mild behavioural consequences than perinatal administration of cannabinoids. This may be due to the relatively low intrinsic efficacy of AEA, the relatively low increase in fetal acyl amides, or that the elevation of other acyl amides may have a protective function.

In summary, we found that perinatal inhibition of FAAH does not affect pup survival, growth or gross brain anatomy. It also does not affect general measures of anxiety, sensorimotor integration or motor learning. However, it appears to have modest effects on learned helplessness, working memory and passive reward. These findings should be considered if FAAH inhibitors are developed for clinical use in a population with childbearing potential.

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Conflict of interest

None.

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